Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 393–399
Published online 8 May 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.858

JOURNAL OF PEPTIDE SCIENCE

Characterization of the branched antimicrobial peptide M6 by analyzing its mechanism of action and *in vivo* toxicity

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Received 21 December 2006; Revised 22 February 2007; Accepted 1 March 2007

Abstract: We analyzed functional activity of the antimicrobial peptide M6 *in vitro* and *in vivo*. The peptide was identified by our group by phage library selection, rational modification and synthesis in a tetrabranched form (Pini *et al.*, *Antimicrob. Agents Chemother.* 2005; 49: 2665–72). We found that it binds lipopolysaccharide, causes perforation of cell membranes without destroying external cell morphology and strongly binds DNA. The latter feature suggests that it could inhibit metabolic pathways, blocking DNA replication and/or transcription. We also observed that M6 does not stimulate humoral immune response when repeatedly administered to animals. We also analyzed M6 toxicity when administered to animals by intraperitoneal or by intravenous injection, determining a preliminary LD50 (125 and 37.5 mg/kg, respectively), which suggested that M6 could be used *in vivo*. These features make the antimicrobial branched peptide M6 a promising candidate for the development of a new antibacterial drug. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptides; branched Multiple Antigen Peptide; peptide stability; peptide *in vivo* toxicity; peptide internalization

INTRODUCTION

Bacterial diseases are currently a leading cause of death in developed countries. A key contributing factor to the alarming increase in bacterial infections is the prevalence of antibiotic resistance, which is expected to continue rising in the next few years [1]. Antibiotic resistance has been detected in the hospital-based population within 6 months of introduction of a new antibiotic, and drug-resistant strains are also becoming very common in the community [2], demonstrating a clear need for a new class of antibiotics.

Antimicrobial peptides are a family of antibiotics that have stimulated research and clinical interest [3–4]. Most antibacterial peptides are components of the innate immunity of animals, plants and microorganisms to microbial infections [5–7]. They generally have a positive net charge [8–9] and seem to interact selectively with anionic bacterial membranes [10–12], although different peptides may have different bactericidal mechanisms [13]. For an exhaustive review see Ref. 14.

The use of peptides, *in vivo*, has largely been limited by their short half-life, since peptides are generally quickly hydrolyzed by endogenous proteases and peptidases. Peptide half-life is, therefore, a bottleneck in the development of new peptide drugs.

In a previous study [15] we reported that synthesis of bioactive peptides in branched form (multiple antigen peptide: MAP) can increase half-life due to acquired resistance to protease and peptidase activity. These branched molecules, first developed in the 1980s [16–17], have a peptidyl core of radially branched lysine residues onto which peptide sequences can be added using standard solid-phase chemistry.

We recently reported the identification and characterization of tetrabranched antimicrobial peptides selected from a large 10-mer phage peptide library and subsequently modified for stability and activity [18]. A brief summary of that work is reported in the results section.

The minimal inhibitory concentrations (MICs) of the most potent peptide, M6, were as low as 4–8 μg/ml against clinical isolates of multi-drug resistant gram-negative bacteria such as *Pseudomonas aeruginosa* and *Enterobacteriaceae*. The same branched peptide showed high stability to blood proteases, low hemolytic activity and low cytotoxic effects on eukaryotic cells.

Here, we focused on biological activity of the branched peptide M6, demonstrating that it binds *E. coli* LPS and enters cells after a few minutes of incubation. It also binds DNA strongly, suggesting biological action inside cells. In view of clinical development of M6, a preliminary study on *in vivo* toxicity and immunogenicity was also carried out. These results, along with the characteristics reported previously, make M6 a promising candidate for the development of a new antibacterial drug.

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MATERIALS AND METHODS

Peptide Synthesis

Monomeric peptide was synthesized as peptide amide by an automated synthesizer (MultiSynTech, Witten, Germany) on a Rink Amide MBHA resin (Nova Biochem) using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/ 1,3-diisopropylethylamine activation. Branched peptide molecules, MAPs, were synthesized on $Fmoc_4$-Lys_2-Lys-β-Ala Wang resin. Side chain protecting groups were tert-butyl ester for Glu, trityl for Gln, tert-butoxycarbonyl for Lys, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg, and tert-butyl ether for Ser. Peptides were then cleaved from the resin and deprotected with trifluoroacetic acid containing water and triisopropylsilane (95/2.5/2.5). Crude peptides were purified by reversed-phase chromatography on a Vydac C18 column. Identity and purity of final products was confirmed by Ettan MALDI-TOF mass spectrometry (Amersham Biosciences). Biotin- and rhodamine-labeled peptides were synthesized using respectively Lys-biotin and Lys-tetramethylrhodamine (Lys-TMR) as first aminoacids.

E. coli Antibacterial Activity

Peptide diluted in 10 mM sodium phosphate buffer (pH 7.4) was added (25 µl) to 25 µl of early growing TG1 *E. coli* cells (final concentration 8×10^7 colony-forming unit (CFU)/ml) in 2 × Ty medium (16 g/l tryptone, 10 g/l yeast extract and 5 g/l Nacl, pH 7) and the mixtures were incubated at 37 °C for 75 min. CFU counts were then determined by plating dilutions of each mixture in 2 × YT-agar medium. A test without added peptide was always carried out in parallel as control.

DNA Binding Assay

Gel-retardation experiments were performed by mixing 200 ng of the *E. coli* plasmid vector pCEP4 (Invitrogen) with increasing amounts of M6 peptide in 20 µl of binding buffer (5% glycerol, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 20 mM KCl and 50 µg/ml BSA). The reaction mixtures were incubated at room temperature for 1 h. Subsequently, 4 µl of native loading buffer was added (40% sucrose, 0.25% bromophenol blue) and an aliquot of 12 µl was run 1% agarose gel electrophoresis in 1 mM Tris borate-EDTA buffer.

Surface Plasmon Resonance Analysis

Biotinylated monomeric QKKIRVRLSA peptide was immobilized on an SA Sensor chip (BIACORE AB, Sweden), previously conditioned with 3 min pulse of 1 M Nacl-50 mM NaOH, obtaining 1600RUs. Several concentrations of LPS (from E.coli O111 : B4, Sigma), diluted in HBS buffer (0.3 M Nacl, 10 mM Hepes, 0.45 mM EDTA, 0.05% P20 pH 7.5), was injected for 4 min at a flow rate of 10 µl/min over the matrix on which QKKIRVRLSA peptide sequence had previously been immobilized. Dissociation was performed for 400 s at the same flow rate. Regeneration was performed with a minute pulse of 30 mM HCl. Association and dissociation kinetic rate constants (*k*on and *k*off) and the equilibrium dissociation constant *K*d were calculated using BIAevaluation 3.0 software.

Confocal Laser-Scanning Microscopy

TG1 *E. coli* cells were grown overnight in 2 × TY. After dilution 1 : 10 in cell medium, 5 × 1 ml aliquots were prepared, washed twice with 10 mM phosphate buffered saline (PBS) pH 7.4 and incubated in 200 µl of a TMR labeled peptide solution (20 µg/ml in PBS) for 5 min at 37 °C. After washing with PBS, each aliquot of cells was resuspended in 200 µl PBS and kept in the dark at 37 °C for 2, 30, 60, 120, 240 min. The cells were then mounted on a glass slide and observed with a Bio-Rad MRC600 confocal laser scanning microscope (CLSM). Fluorescent images were obtained with a 568-nm bandpass filter for excitation of TMR.

The double-staining method was performed as follows: *E. coli* cells were prepared as described above and treated with 5, 10, 20, 40 µg/ml M6 peptide for 30 min at 37 °C. The cells were then washed with PBS, and a FITC solution (6 µg/ml in PBS) was added. After 30 min at 37 °C, the FITC solution was removed and the cells were washed again with PBS. A PI solution (6 µg/ml in PBS) was then added to the cells. Fluorescent images were obtained with a 568-nm bandpass filter for excitation of TMR and with a 488-nm bandpass filter for FITC. Software merging of images was carried out using COMOS software.

Peptide Processing in Serum and Plasma

Eight micro litres of a 1-mg/ml solution of peptides was incubated at 37 °C with 20 µl human serum or plasma. Samples withdrawn after 2, 5 and 24 h were precipitated with 200 µl methanol, centrifuged for 1 min at 10 000 × g and diluted with 800 µl 0.1% TFA in water. These solutions were analyzed by HPLC using a C18-column. Controls for peptide retention time in the crude mixture were obtained by adding the same concentration of peptides to supernatants of plasma or serum treated with methanol and centrifuged as above, running the mixture immediately. MS analysis of the supernatant of crude solutions was performed on an ETTAN MALDI TOF mass spectrometer.

Immunogenicity

Over a 36-day period, Swiss mice (25 g each) were injected intraperitoneally with 1 mg M6 peptide on days 1–7, 15–21 and 28–35. On days 0 and 18, drops of blood were collected by cheek puncture. On day 36 mice were sacrificed and blood collected. ELISA tests were performed immobilizing M6 peptide in plastic wells directly and via streptavidin coated plates and biotinylated M6. Wells were blocked with 3% PBS-BSA for 2 h. Hundred microlitres µl serum was incubated for 1 h at 1 : 50, 1 : 100 and 1 : 500 dilutions in 3% PBS-BSA. Anti-mouse IgM and anti-mouse IgG peroxidase conjugates (Sigma, respectively A8786 and A2554) diluted as suggested by the supplier, were incubated for 1 h. Colorimetric reaction was developed with peroxidase substrate (phosphocitrate, TMB and H2O2) and blocked with 50 µl HCl 1 m.

In Vivo Toxicity

M6 doses ranging from 1 to 4 mg for intraperitoneal administration and ranging from 0.25 to 1.5 mg for intravenous injection were administered at a concentration of 2 mg/ml to

DOI: 10.1002/psc

Swiss mice (20 g each). Mice were checked for symptoms for 7 days.

RESULTS

Peptide Identification and Antibacterial Activity Analysis

Phage library construction, specific selection against whole *E. coli* cells and first characterization of peptide activity is described in Ref. 18. Briefly, selection provided the sequence QEKIRVRLSA which showed the typical amphipathic profile of antimicrobial peptides. This sequence was synthesized in monomeric (L1) and tetrabranched MAP form (M1). Antibacterial activity of the tetrabranched peptide M1 was much higher than that of the monomeric form but M1 was unstable in solution. The M1 peptide sequence was consequently modified to produce the following peptides synthesized as monomers and tetrabranched MAPs: QAKIRVRLSA (M4), KIRVRLSA (M5), and QKKIRVRLSA (M6). These peptides showed high stability to prolonged storage and better antimicrobial activity on TG1 *E. coli* cells than M1 (Figure 1) when synthesized in tetrabranched MAP form. Tetrabranched MAP peptides were particularly resistant to blood proteases and peptidases. The MICs of the most potent peptide, M6, were as low as 4–8 μg/ml against clinical isolates of multi-drug resistant *P. aeruginosa* and *Enterobacteriaceae* [18].

Binding to LPS

In order to test M6 binding to LPS, we exploited Surface Plasmon Resonance using a BIACORE 1000 upgraded instrument. LPS from *E. coli* was diluted in HBS and injected at a flow rate of 10 μl/min over a BIACORE flow cell where biotinylated monomeric peptide had previously been captured via streptavidin. Kinetic analysis, calculated with BIAevaluation software, revealed good binding parameters with a KD of 3.17e–9. LPS injected at the same concentration over an empty flow cell gave no signal, confirming the specificity of the binding. Binding of LPS to immobilized peptide was not sensibly modified by increasing ionic strength of the running buffer (300 mM Nacl) (Figure 2). The fact that LPS binds to the peptide at relatively high ionic strength supports the notion that hydrophobic interactions contribute significantly to the stability of interactions between the side chain of bound peptides and lipid A.

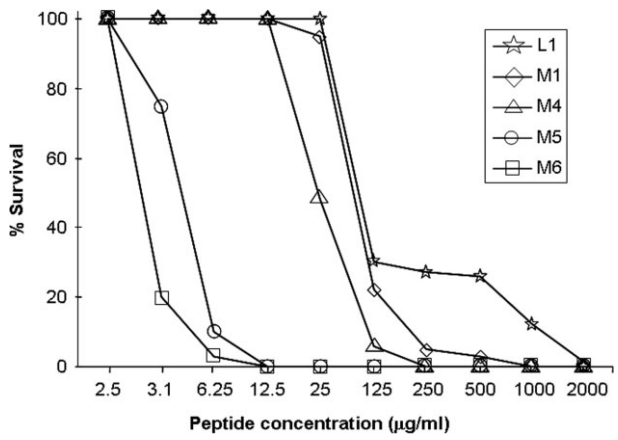

Figure 1 Antibacterial activity of monomeric peptide L1 and tetrabranched peptides M1, M4, M5 and M6. Experiments were performed incubating *E. coli* TG1 cells (8×10^7 CFU/ml) with the indicated amounts of peptides. The survival percentage is the number of living colonies with respect to the number of colonies in controls without peptides.

Visualization of Permeation

A first visualization of membrane perforation by M6, performed by CLSM with rhodamine-labeled branched peptide (red color), showed that labeled M6 entered *E. coli* cells after 5 min of incubation and clustered in discrete patches, often situated at the cell poles, instead of distributing evenly inside the bacteria (Figure 3). There were no significant differences between *E. coli* images taken after 5 (Figure 3(a)) and 240 min of incubation with 20 μg/ml M6 (Figure 3(b)), a concentration which causes 100% reduction in CFU (check Figure 1).

To further visualize membrane perturbation by M6, an additional permeation test was performed using a double staining system involving free FITC (green color) and free propidium iodide (PI; red color). These two dyes do not stain nonpermeated cells (not shown). *E. coli* cells were incubated with 5 μg/ml (Figure 4(a)) and 40 μg/ml of M6 (Figure 4(b)). After 30 min of incubation, cells were incubated with both dyes. At the lowest concentration of M6, *E. coli* cells were only permeated by PI dye (Figure 4(a)). At the highest peptide concentration, *E. coli* cells were permeated by both dyes, as shown by the yellow color due to the sum of red plus green (Figure 4(b)). M6 MIC for *E. coli* is around 5–10 μg/ml as reported in 18. Since FITC, which is a negative charged dye in solution and smaller than PI

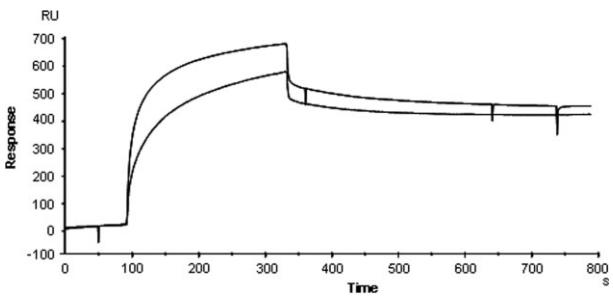

Figure 2 BIACORE sensorgrams obtained by flowing LPS at 2.5 (lower line) and 5 (upper line) μg/ml in HBS 300 mM Nacl over biotinylated QKKIRVRLSA peptide. After 400 s of dissociation, LPS binding to peptide was >350 RU.

DOI: 10.1002/psc

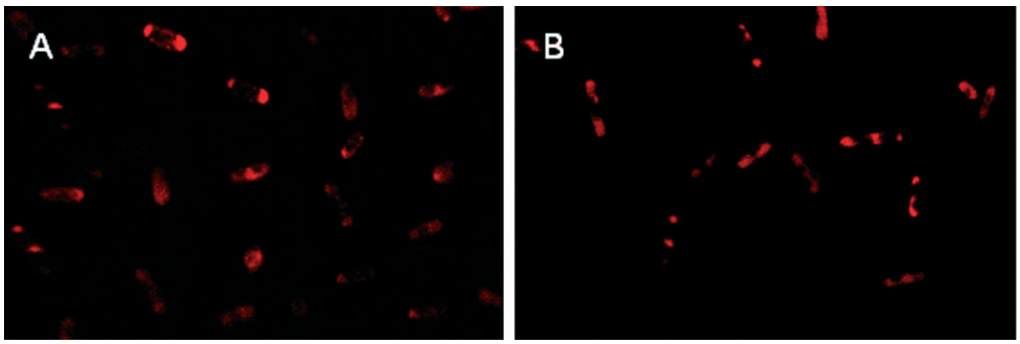

Figure 3 CLSM image of TG1 *E. coli* cells treated with rhodamine-labeled M6 after 5 min (**A**) and 240 min (**B**) of incubation.

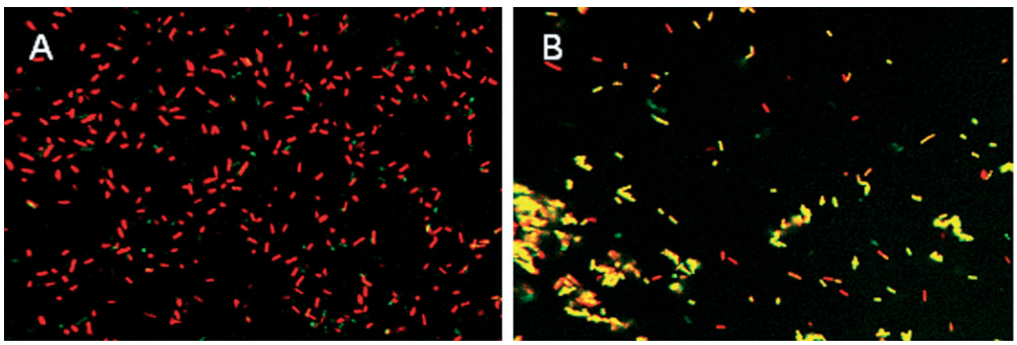

Figure 4 Detection of membrane-damaged bacteria using double staining with FITC and PI fluorescent probes. *E. coli* cells were incubated with 5 μg/ml (**A**) and 40 μg/ml (**B**) of M6. After 30 min of incubation, cells were incubated with both dyes.

(389.4 Da *versus* 668.4 Da), did not enter the cells at 5 μg/ml, this suggests that membranes are only slightly damaged by M6 at its MIC. This indicates that membrane perturbation by M6 is not necessarily the only mechanism of bacterial death. Furthermore, it is of interest to note that all treated bacteria maintained the typical rod shape and did not lose their nucleic acids, even at the highest concentration, as manifested by their clear, intense red fluorescence due to PI binding to DNA.

DNA Binding

In an attempt to clarify the molecular mechanism of action, we examined the DNA binding properties of M6 branched peptide. Its DNA binding was examined by analyzing the electrophoretic mobility of DNA bands (commercial pCEP4 plasmid vector) at the various weight ratios of peptide to DNA on an agarose gel (1%, w/v) following protocols already used for different antimicrobial peptides [19]. M6 inhibited the migration of DNA above weight ratio of 0.2 (Figure 5). This result is of interest because the antimicrobial peptide Buforin II, which is considered a cell function inhibitor by virtue of binding to DNA and RNA, inhibits experimental DNA migration above a weight ratio of 4 [19]. This result, along with the information that cells do not lose DNA when treated with M6 (Figure 4), suggests that M6 strongly inhibits cell metabolism by repressing DNA synthesis and/or transcription.

Immunogenicity

In order to evaluate its possible immunogenicity, the M6 peptide was administered repeatedly to four Swiss mice according to a 5-week immunization protocol. 1 mg/day of M6 was injected intraperitoneally the first, third and fifth weeks. Blood was collected for analysis before immunization, at mid-time and at the end of treatment. Serum was tested by ELISA for IgG and IgM in M6-coated plastic wells and also on an unrelated MAP in order to test for stickiness of serum on immobilized MAP molecules. The unrelated peptide was a tetrabranched peptide of the same length. This experiment was performed with different coating conditions and serum dilutions (Material and Methods). All experiments gave the same result. Figure 6, showing the results obtained with 100 μg/ml M6 coating and 1 : 50 serum dilution, shows that the mice did not raise a detectable antibody response.

In Vivo Toxicity

Preliminary acute toxicity of purified M6 peptide was tested by intraperitoneal and intravenous administration. Table 1 shows results from intraperitoneal administration. This experiment suggests that when the molecule is administered nonsystemically, the LD50 is between 2 and 3 mg per mouse and therefore around 125 mg/kg. Table 2 shows results from intravenous

DOI: 10.1002/psc

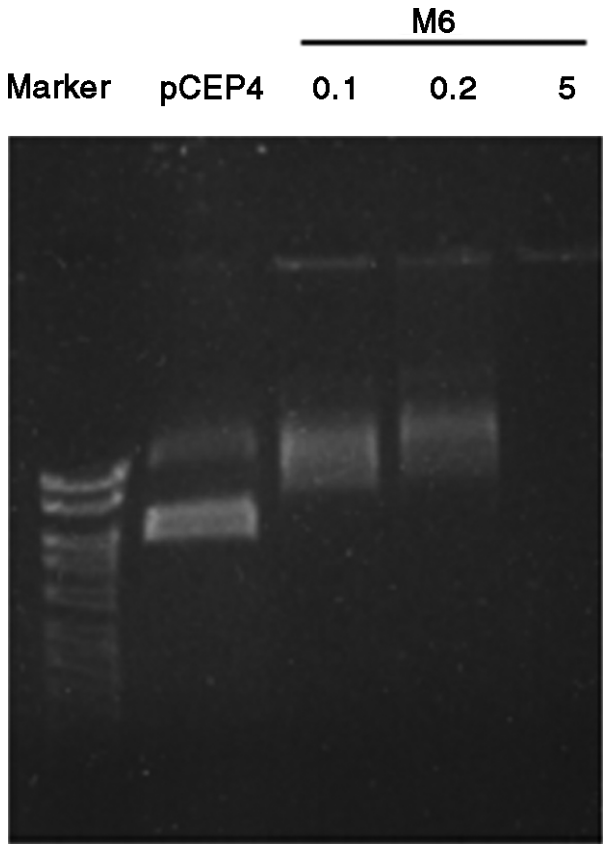

Figure 5 Gel retardation assay. Binding was assayed by the inhibitory effect of peptides on the DNA migration. Various amounts of M6 peptide were incubated with 200 ng of plasmid vector pCEP4 at room temperature for 1 h and the reaction mixtures were applied to a 1% agarose gel electrophoresis. The weight ratio (peptide : DNA) is indicated above each lane.

administration. This experiment suggests that the LD50 is around 0.750 mg per mouse and hence around 37.5 mg/kg when administered systemically.

Stability in Plasma and Serum

We previously reported that the synthesis of bioactive peptides in branched form can result in increased half-life due to acquired resistance to protease and peptidase activity [15,18,20]. Here, we confirmed the increased stability of M6 to proteases and compared it to its linear monomeric analogue (L6) and to two well-known potent monomeric antimicrobial peptides

Table 1 Intraperitoneal administration

Number of mice	M6 dose (mg)	Outcome
4 (around 20 g each)	3	Dead in 48–72 h
4 (around 20 g each)	2	Alive after 7 days

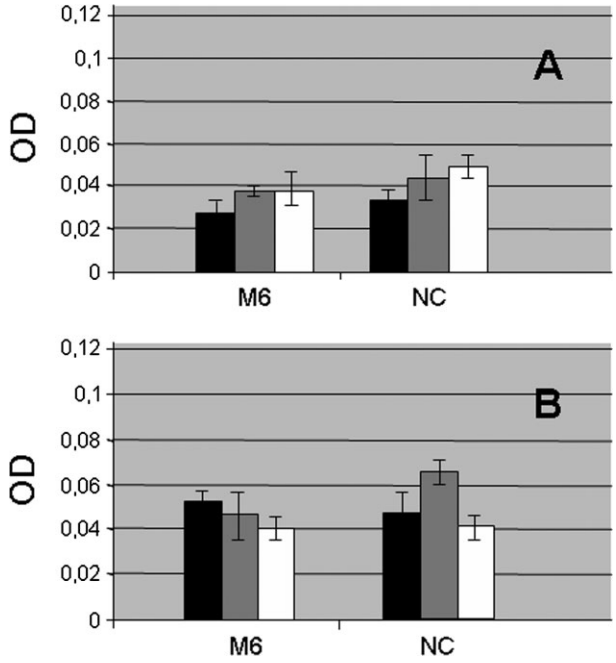

Figure 6 ELISA testing IgM (**A**) and IgG (**B**) in the serum of mice repeatedly injected with 1 mg of peptide M6. Serum was collected before the injections (black columns), at mid-period (grey columns) and at the end (white columns) of the injection period (5 weeks). Serum derived from immunized mice was analyzed on M6 and on unrelated (NC) MAP peptides immobilized on plastic ELISA wells.

Table 2 Intravenous administration

Number of mice	M6 dose (mg)	Outcome
2 (around 20 g each)	1.5	Dead in 1 h
2 (around 20 g each)	1	Dead in 1 h
2 (around 20 g each)	0.75	1 dead after 4 h, 1 alive
2 (around 20 g each)	0.5	Alive after 7 days
2 (around 20 g each)	0.25	Alive after 7 days

Indolicidin (ILPWKWPWWPRR) and Magainin II (GIGKFLHSAKKFGKAFVGEIMNS).

The tetrabranched peptide M6 was stable for 24 h in plasma and in serum, whereas linear peptides L6 and Magainine II were still present in plasma after 2 h, but were no longer detectable after 2 h incubation in serum and 24 h in plasma. Indolicidin was still detectable in plasma and serum after 2h, but was cleaved in less than 24 h in plasma and serum (Table 3).

The stability of M6 to purified proteases trypsin and chimotrypsin was also assayed. It is cleaved in less than 2 h with both enzymes (not shown).

DOI: 10.1002/psc

Table 3 Comparison of peptide resistance to blood proteases

Peptide	Plasma		Serum	
	2 h	24 h	2 h	24 h
M6	+	+	+	+
L6	+	−	−	−
Magainin II	+	−	−	−
Indolicidin	+	−	+	−

DISCUSSION

Today, thanks to modern combinatorial biology, random peptide libraries can also be considered a source of ligand molecules to bind a specific target. The concept of 'magic bullet', initially ascribed to immunoglobulins by Paul Ehrlich at the beginning of the 20th century and strengthened by the hybridoma technology of Kholer and Milstain in the mid 70s, can now also be attributed to peptides. This attribution is increasingly valid in light of the explosion of new technologies for peptide library construction and screening, *in-vivo* peptide stability and peptide conjugation, which make peptide molecules specific projectiles for targeting pathological markers and pathogens [21]. Today, hundreds of peptides are being developed and dozens are in clinical trials for a variety of diseases [22] demonstrating that the general reluctance towards peptide drugs that existed a decade ago, has now been overcome. In spite of this progress, the development of new peptide drugs has largely been limited by their short half-life.

We previously reported that the synthesis of peptides in the form of branched MAP molecules increases their stability to proteases and peptidases [15,18,20]. Synthesis of peptides in MAP form, after selection from a phage library as in the case of M6 peptide, offers several advantages. First, synthesis in MAP form of sequences selected from a phage library enables the activity of phage peptides to be retained. This may be due to similarities between the structural arrangements of peptides in the MAP and in the phage-exposed forms. In a MAP molecule, peptide sequences are linked to the lysine core by their *C*-terminus, as when they are expressed on the phage fusion protein. Moreover, since MAPs contain more peptide copies, they enable multivalent binding, increasing binding efficiency, like in phage-peptides.

In a previous article, we reported that phage display technology, residue substitution procedures and chemical MAP synthesis enabled us to identify a new peptide sequence (QKKIRVRLSA) with strong antibacterial activity. We demonstrated that this molecule had weak hemolytic activity, low toxicity towards eukaryotic cells, stability to protease degradation and a low MIC for several multi-drug resistant pathogenic gram-negative bacteria [18].

Here, we analyzed some aspects of the mechanism of action of M6 and its toxicity when injected in animals. The first new feature we revealed is that M6 binds bacterial LPS. This is not surprising because LPS binding is a part of self promoted uptake, well known for cationic peptides [9]. However, this aspect is of great interest because binding to soluble LPS suggests detoxifying activity of M6 when toxic LPS is released into the blood stream as a consequence of systemic infections which cause sepsis. LPS binding by M6 may also be considered as one of the factors contributing to M6 low activity against a number of gram-positive bacteria [18], which, constitutionally, do not have LPS. However, M6 binding to LPS may not be directly related to its capacity to enter cells, but rather to its direct interaction with negatively charged bacterial membranes, as hypothesized for other amphipathic antibacterial peptides with a net positive charge.

Experiments of membrane perturbation visualized by CLSM demonstrated that M6 enters cells without destroying the cell body. After prolonged incubation of cells with M6, the bacterial cell body still retained its normal shape. We showed that membrane perforation by M6 did not provoke DNA leakage from cells even after 30 min of incubation (Figure 4). This characteristic, along with the demonstration that M6 strongly binds DNA, suggests that one possible antimicrobial mechanism of action, in addition to membrane perturbation, is related to inhibition of metabolic pathways by reducing or blocking DNA replication and/or transcription.

In view of evaluation of M6 therapeutic activity and, therefore, of a repeated administration in individuals, we also tested its possible antigenic properties. We demonstrated that this molecule does not stimulate production of IgG or IgM after high doses of M6 were repeatedly injected in mice without adjuvants over a 5-week period. Actually, branched peptides such as MAP molecules were invented and have been extensively tested to reproduce single epitopes to stimulate the immune system for new vaccine discovery [17]. However, as widely reported in the literature (for a review see [23]), it is very unlikely that MAP molecules composed of short peptide sequences can elicit an antibody response, if not administered along with adjuvants.

As a further characterization, we also preliminarily tested the *in-vivo* toxicity of M6 in order to identify the maximum dose exploitable in experiments on therapeutic activity. Toxicity was evaluated by intraperitoneal injection and by intravenous administration. The LD50 was about 125 mg/kg and 37.5 mg/kg, respectively. These preliminary values are particularly promising when compared with the LD50 of mammalian antimicrobial peptides, such as cathelicidins, that are considered among the most promising antibacterial

DOI: 10.1002/psc

peptides for clinical development, which have much higher *in vivo* toxicity [24].

In our previous work we demonstrated that M6 peptide has potent antibacterial activity against a panel of gram-negative bacteria, comprising some multi-drug-resistant pathogens. Data on M6 biological activity described here, in addition to the characteristics already reported, make this branched peptide a strong candidate for the development of a new antibacterial drug.

Acknowledgements

This work was supported by the University of Siena (PAR Progetti 2005 to AP and LB) and the Monte Paschi Foundation (2006 to AP).

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